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(71) Applicant *(for all designated States except US)*: EKINS, Roger, Philip [GB/GB]; Department of Molecular Endocrinology, The Middlesex Hospital Medical School, Mortimer Street, London W1N 8AA (GB).

(71)(72) Applicant and Inventor: DAKUBU, Salifu [GH/US]; 19 Belknap Terrace, Winchester, MA 01890 (US).

(74) Agent: HALE, Stephen, Geoffrey; J.Y. & G.W. Johnson, Furnival House, 14-18 High Holborn, London WC1V 6DE (GB).

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(54) Title: LABELLED AND LABELLABLE REAGENTS FOR FLUOROMETRIC ASSAYS

(57) Abstract

Labellable reagents for fluorometric assays comprise a cyclic condensation product of a β-diketone, an aldehyde and an NH_2 -bearing macromolecule, for example an antigen or antibody or a substance having an active group to which an antibody or antigen is linked. The reagents can be chelated to lanthanide metal ions such as Eu (III) and Tb (III) to form fluorescing complexes which can be used as labelled reagents for fluorometric assay of organic substances, for example antigens, antibodies and other substances occurring in body fluids.

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Labelled and labellable reagents for fluorometric assays

Technical Field

This invention relates to the assaying of organic substances found in human and animal body fluids and the like. More particularly it relates to a fluorometric immunoassay and substances for use as labelled reagents in such an assay.

Background Art

There are now a number of applications in which it is required to label antibodies (or other organic substances, for example macromolecules such as proteins, and haptens) with metal ions, either radioactive metal ions for use in radioimmunossay and other nuclear medicine studies or lanthanide metal ions for fluorometric immunoassay and other studies involving fluorescence. For these purposes the organic substances are conventionally labelled with metal ions through the agency of chelates. Hitherto the chelates have been modified with bridging reagents which convert them into bifunctional reagents so that they retain their chelation function whilst being readily attachable by covalent bonding to the molecule to be labelled.

One class of chelating reagents which has been used for this purpose is the class of β-diketones, such as trifluoroacetylacetone and benzoyl and α- and β-naphthoyl trifluoroacetone, and chelates of lanthanide metal ions with such reagents have been coupled to antibodies using EDTA-analogues (see European patent application No. 0,064,484). It has also been proposed in GB Patent Specification No. 1,560,402 to modify a β-diketone ligand such as thenoyl-trifluoroacetylacetone by the attachment

of an aminomethyl substituent and to use one molecule of the modified ligand and two molecules of unmodified ligand to form a lanthanide metal ion complex which readily couples to an antibody after conversion of the amino group to an isothiocyanate group.

However, bifunctional-chelating agents are difficult to synthesize and many of the reactions by which covalent bonding of the ligand-metal ion complex to the molecule to be labelled is achieved have only a low yield of the desired labelled product and may also confer undesirable properties on the labelled molecule.

Disclosure of invention

The present invention seeks to provide an alternative method of achieving satisfactory linking of a metal ion complex to an organic molecule and thereby conferring specialized functionality to the labelled molecule.

According to the present invention a method of potentiating an NH_2-bearing macromolecule for labelling with a metal ion comprises reacting the NH_2-bearing macromolecule with a β-diketone in the presence of an aldehyde to form a cyclic condensation product.

The invention also provides a method of potentiating an organic macromolecule for labelling with a metal ion, comprising reacting an NH_2- bearing compound having an active group capable of reaction with the organic macromolecule with a β-diketone in the presence of an aldehyde to form a cyclic condensation product and linking the resulting product to the organic macromolecule via the active group.

Further it provides a substance capable of being labelled with a metal ion for use in a fluorometric assay technique, comprising a cyclic condensation product

an NH_2-bearing macromolecule, a β-diketone and an aldehyde.

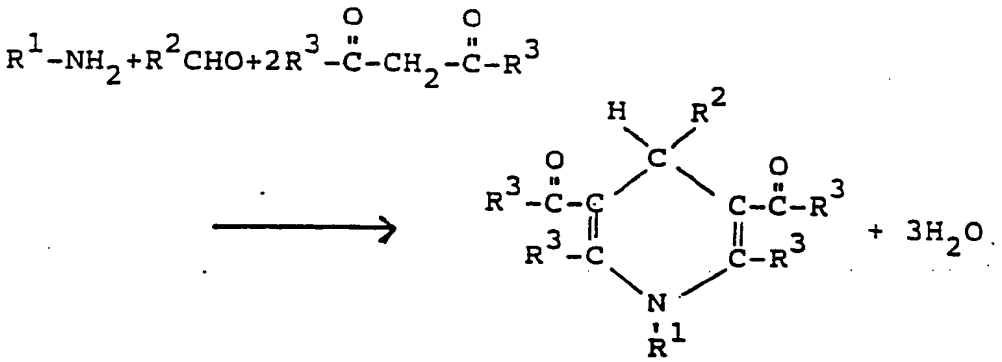
It also provides a labelled reagent for use in fluorometric assay comprising a cyclic condensation product of an aldehyde, a β-diketone and an organic substance having an NH_2 group, the condensation product being chelated to a lanthanide metal ion to form a complex capable of being estimated fluorometrically, and a process for the fluorometric assay of an organic substance in which such a labelled reagent is used.

Brief description of the drawings

The invention is illustrated by the accompanying drawings which are graphs relating to assays of alpha-faeto-protein (selected as using a typical NH_2-containing antibody), as described in the Examples below. Figure 1 is a calibration curve using rabbit anti-alpha-faeto-protein (AFP) directly labelled with trifluoroacetylacetone. Figure 2 is a calibration curve in which the anti-AFP has been labelled using labelled polylysine, and Figure 3 is the calibration curve shown in Figure 2 when Eu(III) fluorescence was fully enhanced.

Detailed description of the invention

The reaction between the NH_2-bearing substance or macromolecule (R^1-NH_2), the β-diketone ($R^3COCH_2COR^3$) and the aldehyde (R^2-CHO) to form the cyclic condensation product can be represented by the following equation:



The symbols R^1, R^2 and R^3 are arbitrary symbols dependent on the nature of the starting materials.

The method of the invention does not require the prior modification of the β-diketone and, because it depends only on the basic structure of the β-diketone and not on the nature of any substituents, it can be used with a wide variety of different β-diketones. It is therefore possible to choose the optimum β-diketone on the basis of other criteria, for example formation of soluble complexes having satisfactory absorption spectra and formation constants with a metal ion introduced for labelling purposes without loss of immuno reactivity or for the avoidance of toxic effects. β-Diketones which have been used successfully include both aromatic and non-aromatic species, and it is preferred to employ a fluorine-substitued material when producing a complex for labelling with a lanthanide metal ion for fluorometric purposes. Examples of suitable β-diketones are trifluoroacetylacetone, thenoyltrifluoroacetone, 4,4,4-trifluoro-1,(2-furyl)1,3-butanedione and α- and β-naphthoyltrifluroacetone. Other suitable β- diketones are those mentioned in an article by Hemmila et al in Analytical Biochemistry, 137, 335-343 (1984) or in UK Patent Specification No. 1560402 or European Patent Application No. 0,064,484 mentioned above or in European Patent Application No. 0,002,963.

It may be advantageous to employ the β-diketone in the form of a metallic β-diketonate with the metal of interest or with another metal (for example with copper) in order to promote the chelation properties of the final product. If the initial metal is not the metal of interest it can then be displaced with the metal of interest, for example a lanthanide metal, to form the labelled product.

The aldehyde used is preferably formaldehyde when

specialized functionality, e.g. chelation, is not required in the aldehyde, but other aldehydes may be used if desired, for example C_1 -C_4 -alkylaldehydes optionally bearing inert substituents, and this gives a further means of varying the properties of the resulting cyclic condensation product. The aldehyde itself may have chelating properties deliberately built into its chemical structure and these will then assist in the chelation of the metal in the labelled product.

The invention can be used to provide labelled reagents, and precursors therefor, from organic substances whether they initially contain sufficient NH_2 groups for the formation of the cyclic condensation product or not. Where the organic substance contains no or insufficient NH_2 groups initially, a cyclic condensation product may be formed by initial reaction of the β-diketone and the aldehyde using an amine or amino acid or other NH_2-containing compound, for example an NH_2-containing polymer (such as a polylysine), having an active group capable of reacting with the macromolecule. The resulting cyclic condensation product is then reacted with the organic substance to be labelled by a mild reaction through the active group to attach it to the organic substance. An example of such a reaction is the carbodiimide reaction. Alternatively, an active ester can be made, for example the hydroxysuccinimide active ester of poly-L-lysine. Instead of the polylysine it is possible to use a lysine-containing chelate, which will then provide further assistance in chelating the metal in the final product. An example of such a chelate is siderophilin (transferrin).

The invention may thus be used to potentiate for labelling with metal ions a wide variety of organic substances naturally occurring in human and animal body fluids or cultivated or generated artificially, for example antigens, antibodies, hormones (e.g. T4), enzymes and other

proteins and haptens, and all such substances are intended to be encompassed within the term organic macromolecule as used herein. The substances in question will in general have molecular weights in excess of 300 in order to qualify as macromolecules, but substances of lower molecular weight are not excluded. The macromolecule will in general contain linked subunits such as amino acid residues but this is not a requirement.

The reaction to form the cyclic condensation product is preferably carried out at a mildly acid pH, for example 5.5 to 6.5, although alkaline pHs are not ruled out. Preferably it is carried out at a mildly elevated temperature, for example 30-50°C. The use of high temperatures which would damage the NH_2-bearing macromolecule is to be avoided when manufacturing the condensation product for use in a subsequent assay. The use of lower temperatures will reduce the rate of reaction and a prolonged incubation period may be required.

Cyclic condensation products according to the invention are stable and strongly chelate metal ions such as lanthanide metal ions. Thus, they may be reacted to form a complex of Eu(III) or other lanthanide metal such as Tb(III) capable of giving fluorescent complexes and the resulting complex will in general be fluorescent in solution without the need to add exogenous β-diketone. The reaction may be carried out in any known or conventional manner, for example by dialysis against a buffer containing ions of the metal to be incorporated.

The condensation products produced with chelating aldehydes satisfy the coordination capacity of the lanthanide metals more completely than the products from other aldehydes. Such products therefore chelate metals better and are more fluorescent when in the form of lanthanide chelates.

The absorption maximum wave lengths of the condensation products are usually slightly shifted either way, depending on the starting β-diketone, from that of the β-diketone. This shift in wave length and hence excitation maximum may be used as an index of the formation of the condensation product.

The cyclic condensation product labelled with a lanthanide metal ion may be used as a labelled reagent in the fluorometric assay of a wide variety of substances but it is primarily intended for the estimation of organic macromolecules and haptens occurring in body fluids either naturally or as a result of disease or malfunction or the treatment of disease or malfunction, for example antibodies, antigens and other proteins, hormones, enzymes, drugs and viral particles. The fluorometric assay may be carried out using any of the known or standard techniques. Examples of suitable techniques are mentioned in the patent specifications and applications referred to above and in the references mentioned in them. See also Dakubu S., Ekins R.P. et al, "High-sensitivity, pulsed-light time-resolved fluoroimmunoassay" in Practical Immunoassay, W. Butt (Ed)., Marcel Dekker Inc., pages 71-101. The assay may be a qualitative assay, used purely for detection of a possible antibody or other substance, or a quantitative assay, used to estimate the concentration of the substance to be assayed.

One preferred assay procedure is a two-site (sandwich) procedure in which the substance to be assayed (e.g. an antibody or antigen) is reacted initially with its complement (e.g. an antigen therefor or an antibody therefor), suitably whilst the latter is attached to a solid substrate, and the product reacted with a labelled reagent capable of interacting with the bound substance being assayed (e.g. labelled antigen or labelled antibody).

An alternative fluorometric assay involves a methodology akin to a standard radioimmunoassay in which the substance to be assayed (e.g. an antigen or antibody) and

a labelled reagent compete for reactive sites on a complement (e.g. an antibody or antigen) which is conveniently attached to a solid substrate.

When the cyclic condensation product is being used in a fluorometric immunoassay it is possible to carry out the measurement of fluorescence for the determination of the lanthanide metal ion (e.g. Eu(III)) either in solution or when the labelled molecule is attached to a solid support. When the measurement is being carried out in solution the lanthanide metal ion can be extracted from the labelled complex, for example by reaction with added β-diketone (which need not be the same as the β-diketone used to form the complex). The added β-diketone complexes with the metal ion and thereby displaces the condensation product, and the resulting complex can be separated from the condensation product. However, it is an advantage of the present invention that it is not necessary to use such an enhancement solution for the measurement of the fluorescence but that the complex of the condensation product with the lanthanide metal ion is inherently fluorescent so that its fluorescence can be measured directly whilst it is still bound to the reaction product.

For determination of the amount of fluorescence in solid form or in solution improved results may be obtained by addition of trioctylphosphine oxide (TOPO), as mentioned in European Patent Application 0,064,484. This enables the labelled macromolecule to be determined fluorometrically to better than 10^{-10}M concentration.

The cyclic condensation product, suitably in solution in a buffer, may thus form part of a kit for carrying out fluorometric assays, the organic substance participating in the condensation product (either directly or by subsequent reaction with an active group of the NH_2-

bearing substance actually participating) being chosen appropriately depending on the substance being assayed so as to be capable of interacting either with the substance to be assayed or with its complement, for example a suitable antibody or antigen. The kit conveniently includes the ready-chelated complex of a lanthanide metal ion such as Eu (III) or Tb (III) with the condensation product. The kit may also include as a separate component a complement for the substance to be assayed, that complement being bound on a solid support.

Modes for carrying out the invention

The following reagents were used in the following examples which are presented for illustrative purposes only and are not intended to limit the scope of the invention.

1) A commercially available rabbit anti-AFP solution containing approximately 1 mg/ml.

2) A 1:10 dilution of a formaldehyde solution originally 37-40% w/v in formaldehyde, thus now having a concentration of about 1.4×10^{-3} moles/litre.

3) A stock solution of cupric trifluoroacetylacetone (CuTFAA) in methanol at a concentration of 160 mM.

Example 1

400 μl of the anti-AFP solution (i.e. 2.7×10^{-8} mole of anti-AFP) were incubated at 37°C for 1 hour with 200 μl of the diluted formaldehyde solution (i.e. 2.7×10^{-7} mole of formaldehyde) and 4 μl of the Cu TFAA solution (i.e. 5.4×10^{-7} mole of Cu TFAA) in an acetate buffer (0.2 M) at a pH of 5.7. The reaction product was dialysed initially against the acetate buffer to remove

unreacted small molecules then against the acetate buffer containing Eu(III) ions at 10^{-7} M concentration to form the chelation product (labelled antibody), and finally against the acetate buffer to remove excess Eu(III). The labelled antibody was dissolved in a tris(hydroxymethyl) aminomethane/saline/azide buffer (0.02 M) at a pH of 7.6 (50 mM in NaCl) for assay purposes. If desired, it could have been further purified by chromatography.

Example 2

The above procedure was repeated using, in place of the rabbit anti-AFP, a polylysine of molelcular weight 80,000. The resulting labelled polylysine was coupled to antibody using 1-ethyl-3 (3-dimethylaminopropyl)-carbodiimide hydrochloride.

The labelled products obtained in Examples 1 and 2 were used in assays for AFP by a standard two-site ("sandwich") assay procedure.

Claims

1. A substance capable of being labelled with a metal ion for use in a fluorometric assay technique, comprising a cyclic condensation product of an NH_2-bearing macromolecule, a β-diketone and an aldehyde.

2. A substance as claimed in claim 1, wherein the NH_2-bearing macromolecule is an antibody or antigen.

3. A substance as claimed in claim 1, wherein the NH_2-bearing macromolecule is an NH_2-containing compound linked via an active group to an antibody or antigen.

4. A substance as claimed in claim 3, wherein the NH_2-bearing macromolecule is a polylysine or ε-lysine-containing chelate having an active group through which it can be linked to an antibody or antigen.

5. A substance as claimed in claim 1, wherein the β-diketone is a fluorine-substituted compound.

6. A substance as claimed in claim 1, wherein the aldehyde is formaldehyde.

7. A substance as claimed in claim 1, wherein either the β-diketone or the aldehyde has chelating properties.

8. A method of potentiating an NH_2-bearing macromolecule for labelling with a metal ion, comprising reacting the NH_2-bearing macromolecule with a β-diketone in the presence of an aldehyde to form a cyclic condensation product.

9. A process as claimed in claim 8, wherein the macromolecule is an antibody or antigen.

10. A method of potentiating an organic macromole-

cule for labelling with a metal ion, comprising reacting an NH_2-bearing compound having an active group capable of reaction with the organic macromolecule with a β-diketone in the presence of an aldehyde to form a cyclic condensation product and linking the resulting product to the organic macromolecule via the active group.

11. A process as claimed in claim 10, wherein the macromolecule is an antibody or antigen.

12. A labelled reagent for use in fluorometric assay comprising a cyclic condensation product of an aldehyde, a β-diketone and an organic substance having an NH_2 group, the condensation product being chelated to a lanthanide metal ion to form a complex capable of being estimated fluorometrically.

13. A labelled reagent as claimed in claim 12, wherein the NH_2 residue in the cyclic condensation is borne on or linked to an organic macromolecule.

14. A labelled reagent as claimed in claim 13, wherein the organic macromolecule is an antibody or antigen.

15. A labelled reagent as claimed in claim 12, wherein the lanthanide metal ion is europium (III) or terbium (III).

16. A process for the fluorometric assay of an organic substance, wherein a labelled reagent as claimed in claim 12 is used.

17. A process as claimed in claim 16 wherein the assay is carried out using a sandwich technique in which the substance to be assayed is initially reacted with its complement

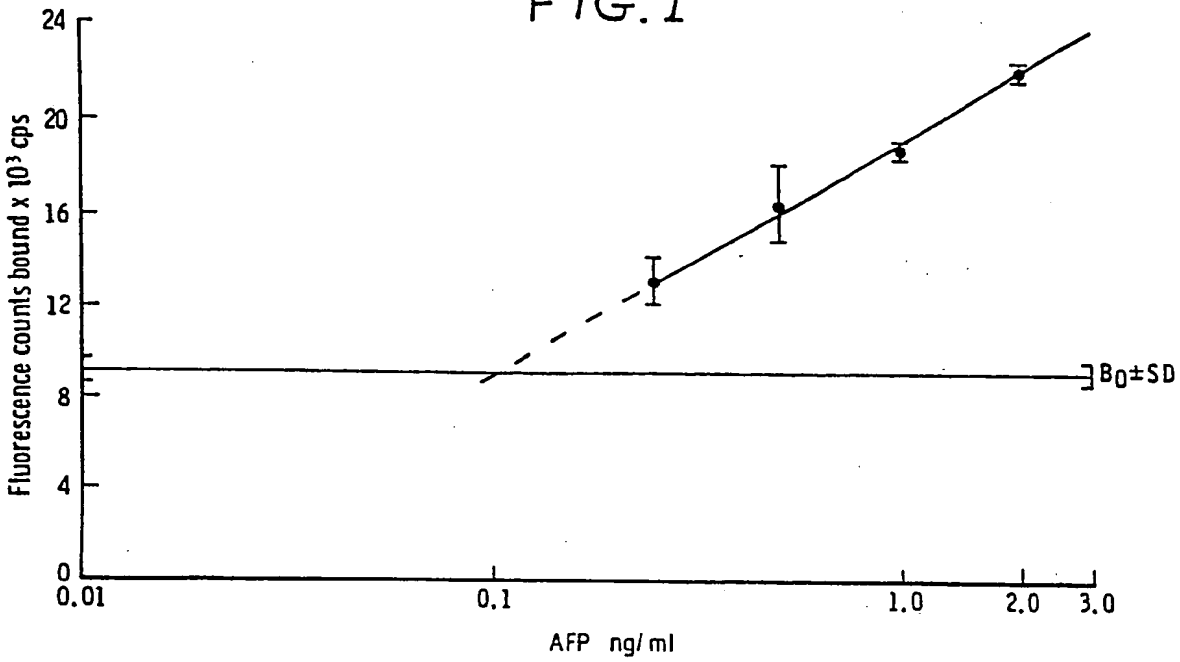
to bind it to the complement and subsequently reacted with a labelled reagent as claimed in claim 12, said labelled reagent including an organic residue capable of interacting with the substance being assayed whilst the latter is bound to the complement.

18. A process as claimed in claim 16, wherein the fluorescence is measured whilst the lanthanide metal ion is still present in the reaction product of the labelled reagent and the substance being assayed and/or its complement.

19. A process as claimed in claim 16, wherein the fluorescence is measured after extraction of the lanthanide metal ion from the reaction product of the labelled reagent and the substance being assayed and/or its complement.

20. A kit for use in the fluorometric assay of an antibody or antigen comprising a solution in a buffer of a cyclic condensation product of a β-diketone, an aldehyde and an organic substance capable of interacting either with the antibody or antigen to be assayed or with its complement, said organic substance participating directly in the cyclic condensation product if it is NH_2-bearing or being linked to an NH_2-bearing compound which participates directly in the cyclic condensation product, the condensation product being chelated with a lanthanide metal ion to form the labelled reagent.

FIG. 1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20
16
12
8
4
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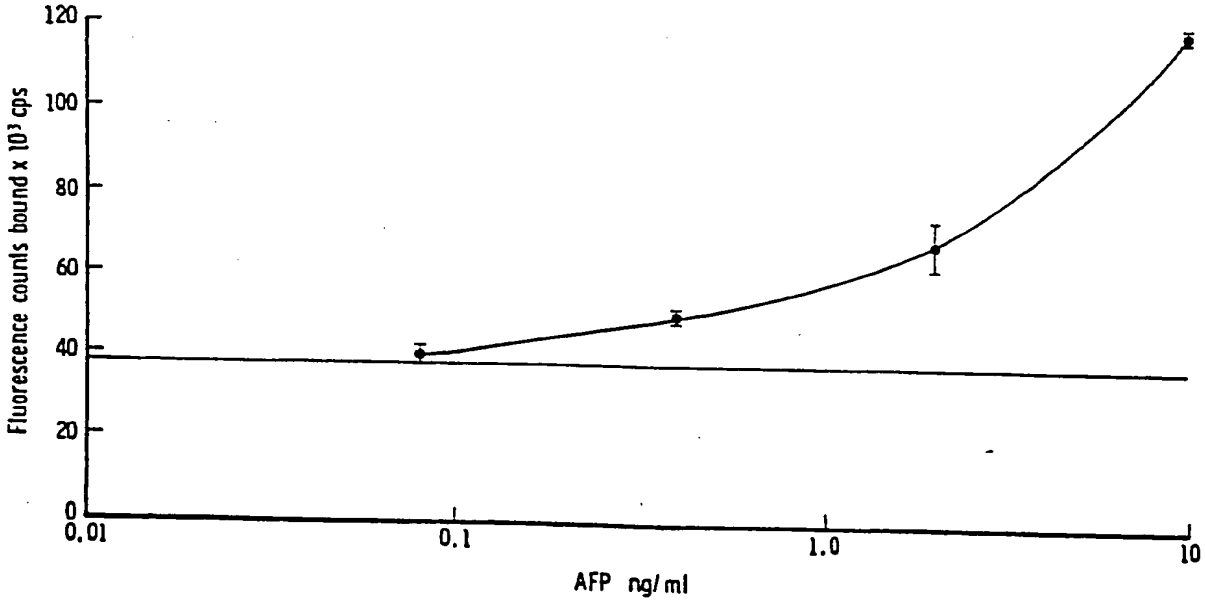
0.1
1.0
2.0
3.0
AFP ng/ml

FIG. 2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1.0
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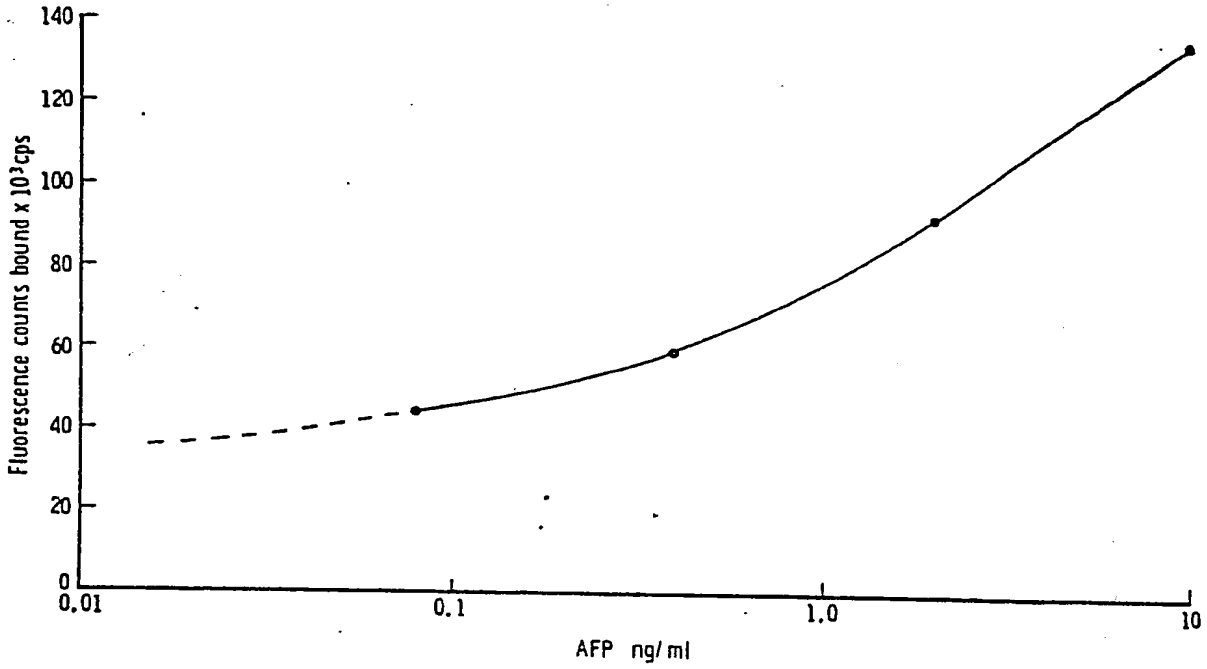
AFP ng/ml

FIG. 3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120
100
80
60
40
20
0
Fluorescence counts bound x 10³cps
0.01
0.1
1.0
10
AFP ng/ml

INTERNATIONAL SEARCH REPORT

International Application No PCT/GB 85/00377

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC[4]: G 01 N 33/533; G 01 N 33/58

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
IPC[4]	G 01 N; C 07 D; C 07 F

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No. [13]
Y	US, A, 4374120 (E. SOINI et al.) 15 February 1983 see claims 1-3,7-9,17	1,8,12
X	see claims 1-3,7-9,12,17	1,2,3,5,7, 9,11,15
Y	FR, A, 2261767 (SMITHKLINE CORPORATION) 19 September 1975 see page 2, lines 25-40; page 3, lines 1-6	1,8,12
A	Clinical Chemistry, vol. 25, no. 3, March 1979 (Winston-Salem, North Carolina, US) E. Soini et al.: "Fluoroimmunoassay: present status and key problems", pages 353-361 see page 359; page 360, column 1	1,5,15
A	Chemical Abstracts, vol. 100, no. 23, 4 June 1984 (Columbus, Ohio, US) I. Hemmila et al.: "Europium as a label in time-resolved immuno-	./.

* Special categories of cited documents: [10]

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IV. CERTIFICATION

Date of the Actual Completion of the International Search	Date of Mailing of this International Search Report
18th November 1985	04 DEC. 1985
International Searching Authority	**Signature of Authorized Officer**
EUROPEAN PATENT OFFICE	G.L.M. Kruydenberg

Form PCT/ISA/210 (second sheet) (January 1985)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
	fluorometric assays", see page 273, column 2, abstract no. 188162q & Analyt. Biochem., 1984, 137, (2), 335-43 --	
A	GB, A, 2060623 (ANALYTICAL PRODUCTION) 7 May 1981 ------	

ANNEX TO THE INTERNATIONAL SEARCH REPORT ON

INTERNATIONAL APPLICATION NO. PCT/GB 85/00377 (SA 10464)

This Annex lists the patent family members relating to the patent documents cited in the above-mentioned international search report. The members are as contained in the European Patent Office EDP file on 26/11/85

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Patent document cited in search report	Publication date	Patent family member(s)		Publication date
US-A- 4374120	15/02/83	SE-B-	428332	20/06/83
		SE-A-	7902079	09/09/80
FR-A- 2261767	19/09/75	BE-A-	824925	29/07/
		DE-A-	2506987	28/08/75
		GB-A-	1438931	09/06/76
		US-A-	3956341	11/05/76
		JP-A-	50117779	16/09/75
GB-A- 2060623	07/05/81	DE-A-	3033691	19/03/81
		JP-A-	56059741	23/05/81
		FR-A,B	2469416	22/05/81
		US-A-	4352751	05/10/82
		US-A-	4432907	21/02/84